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Genotypic Characterization of *Salmonella enteritidis* Phage Types by Plasmid Analysis, Ribotyping, and Pulsed-Field Gel Electrophoresis

A. M. RIDLEY,* E. J. THRELFALL, AND B. ROWE

Laboratory of Enteric Pathogens, Central Public Health Laboratory, London NW9 5HT, United Kingdom

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Pulsed-field gel electrophoresis (PFGE) was used to resolve *Xba*I and *Spe*I macrorestriction fragments from 60 defined phage type (PT) reference strains of *Salmonella enteritidis*. The level of discrimination was compared to that afforded by plasmid profile analysis and ribotyping. Twenty-eight distinct *Xba*I pulsed-field profiles (PFPs) were observed, although a single type, PFP X1, predominated. Absence of the 57-kb *spv*-associated fragment was observed for three PT reference strains, and the profile was designated PFP X1A. The *Xba*I macrorestriction profiles of a further four PT reference strains were altered by the presence of plasmid-associated bands. Twenty-six *Spe*I-generated PFPs (plus one subtype) were observed for the same strains. No *Spe*I fragment corresponding to the 38-MDa serovar-specific plasmid was detected. The distribution of *Xba*I and *Spe*I profiles did not always correspond, producing a total of 32 combined PFPs for the 60 PT reference strains. This compared with a total of 18 different plasmid profiles and three *Pvu*II ribotypes generated by the same strains. The results of this study indicate that PFGE may offer an improved level of discrimination over other genotypic typing methods for the epidemiological typing of *S. enteritidis*.

Salmonella enteritidis remains the most common *Salmonella* serotype recovered from humans in England and Wales (1), with 18,968 isolates referred to the Laboratory of Enteric Pathogens in 1996. Although phage type (PT) 4 has predominated for some time, the number of isolates involving this PT appears to be declining, with a corresponding increase in isolations of PTs 6, 1, 8, and 6a being recorded. Of isolates of *S. enteritidis* received during 1996, five PTs (4, 1, 6, 6a, and 8) accounted for 88%, and specifically, 72% of the strains belonged to PT 4, 5% to PT 6, 4% to PT 1, 3% to PT 8, and 3% to PT 6a.

Strain identification is essential for the effective investigation of common-source outbreaks, and phage typing is the method of choice for the primary differentiation of *S. enteritidis*. The scheme of Ward and colleagues (28) defined 27 PTs for this serovar (22), and this has subsequently been extended to 60 types (28a). However, this method has limited applicability for epidemiological studies, as the majority of isolates from cases of human infection in the United Kingdom since 1987 have belonged to PT 4. Further subdivision by DNA-based methods may therefore be required for investigation of outbreaks. Plasmid profiling has been shown to be of limited use for the subdivision of *S. enteritidis* PT 4, as many strains carry a single 38-MDa plasmid (23, 24). DNA probes based on insertion sequence IS*200* (7) generate only two fragments with the majority of *S. enteritidis* PTs (21), consequently limiting the discriminatory potential. Probes based on rRNA, including rRNA (4, 10), the cloned *rrn* operon of *Escherichia coli* (2), and an intragenic fragment of the 16S *rrn* gene amplified by PCR (18), have been applied to the analysis and elucidation of phylogenetic relationships in several *Salmonella* serotypes. Probes based on known (20) or randomly cloned (25) sequences have also been used with varying degrees of success. These methods have led to the conclusion that *S. enteritidis* falls into three clonal lines, with the prevalent PTs, PT 4 and PT 6, falling in the first group (14).

Pulsed-field gel electrophoresis (PFGE) has become established as a method for the analysis of large DNA fragments generated by restriction endonuclease digestion of genomic DNA (17). The procedure has been used successfully for the estimation of genomic size and the determination of the physical map of *S. enteritidis* SSU7998 (9). PFGE has also recently been applied to epidemiological investigation of *Salmonella* serovars and provides a useful indicator of the level of genotypic diversity existing between strains (12, 14). Preliminary studies have demonstrated that strains of *S. enteritidis* PT 4 can be subdivided by PFGE (15). However, to further evaluate the potential of the method for all *S. enteritidis* PTs, it is important that phylogenetic relationships between the different PTs of *S. enteritidis* be elucidated in relation to the relationships provided by chromosomal fingerprinting methods, such as pulsed-field profile (PFP) patterns and restriction fragment length polymorphism (RFLP) at 16S rRNA gene loci.

We describe the genotypic characterization of *S. enteritidis* PT reference strains by PFGE and ribotyping. The epidemiological applicability of these methods is also assessed.

MATERIALS AND METHODS

Bacterial strains. Sixty strains representing the currently defined PT reference strains of *S. enteritidis*, listed in Table 1, were stored on nutrient agar slopes and grown overnight in nutrient broth at 37°C. The strains were maintained in the culture collection of the Laboratory of Enteric Pathogens and had been phage typed by standard methods (28).

Plasmid analysis. Plasmid DNA was isolated by the method of Kado and Liu (6) and analyzed by agarose gel electrophoresis with *E. coli* 39R861 as a plasmid molecular mass marker (24). Plasmid sizes were determined with reference to 39R861 and the 38-MDa *S. enteritidis* serovar-specific plasmid (SSP). Plasmid DNA was transferred to Hybond N hybridization membranes (Amersham International, Amersham, United Kingdom) by capillary blotting. The presence of the *Salmonella* plasmid virulence (*spv*) region was determined by hybridization to a digoxigenin-labelled probe comprising a 437-bp fragment encoding the *spvC* virulence gene, prepared by PCR with oligonucleotide primers (Boehringer Mannheim, Lewes, United Kingdom) (4a). Detection of the hybridization signal was done according to the manufacturer's recommendations.

* Corresponding author. Mailing address: Laboratory of Enteric Pathogens, Central Public Health Laboratory, 61 Colindale Ave., London NW9 5HT, United Kingdom. Phone: 0181 200 4400. Fax: 0181 905 9929. E-mail: jthrelfall@phls.co.uk.

TABLE 1. Characteristics of the 60 *S. enteritidis* PT reference strains

PT	Reference strain	Plasmid(s) (MDa)	Ribotype (*Pvu*II)	PFGE profile		
				*Xba*I	*Spe*I	Combined
1	E2331	38	A	X11	S2	XS5
1a	P221267		A	X1A	S1	XS1A
1b	P265000	38	A	X11	S2	XS5
1c	P367587	38, 8.0	A	X1	S1	XS1
2	E2457	38	A	X12	S3	XS7
3	P66040	38	A	X13	S4	XS11
4	E2187	38	A	X1	S1	XS1
4a	P99764	38	A	X1	S1	XS1
5	P70001	38	A	X11A	S2	XS5A
5a	P114100	65, 38	A	X1	S1	XS1
5b	P316257	38, 2.8	A	X1	S1	XS1
6	P99327	50, 4.6, 2.6	A	X1B	S5	XS12
6a	E2408	59, 4.0, 3.0	B	X15	S6	XS13
6b	P332932	70, 38	A	X1D	S1	XS1D
7	P102936	38	A	X12A	S3A	XS8
7a	P267687	38	A	X1	S1	XS1
8	E2468	38	A	X12	S3	XS7
8a	P278053	38, 2.8	A	X12	S24	XS33
9	E2402	38	A	X16	S29	XS14
9a	P106583	59	C	X17	S8	XS15
9b	P107739	59	C	X18	S9	XS16
9c	P312425	33	A	X35	S25	XS34
10	E3945	65	A	X32	S10	XS10
11	E2109	59	C	X19	S11	XS17
11a	P138678	38	A	X20	S12	XS18
11b	P187803		C	X21	S13	XS19
12	P95661	38	A	X27	S1	XS25
13	E464	38	A	X22	S14	XS20
13a	P102936	38	A	X12	S3	XS7
14	E2387	59	C	X23	S15	XS21
14b	P118526	38	B	X1	S1	XS1
15	E2402	38	A	X24	S3	XS22
16	E866		B	X25	S16	XS23
17	P95940	38	A	X26	S1	XS24
18	P89448	38	A	X14	S1	XS2
19	E1949	45, 4.2	A	X13A	S4	XS11A
20	P68147	59	B	X28	S11	XS26
20a	P138532	38, 32	A	X29	S17	XS27
21	P72580	38	A	X1	S1	XS1
22	P84357	65, 38	A	X30	S3	XS28
23	P88255	38	A	X12	S3	XS7
24	P99768	38	A	X1	S1	XS1
24a	P310001	70, 38	A	X36	S27	XS35
25	P100613	38, 4.0	A	X1	S1	XS1
26	P106993		B	X31	S18	XS29
27	P122530	38	A	X1	S19	XS3
28	P125363	38, 8.0	A	X12	S3	XS7
29	P124191		A	X1A	S1	XS1A
30	P104204	65, 45	A	X1E	S1	XS1E
31	P130531	38	A	X1	S20	XS4
32	P135293	38, 2.0	A	X11	S2	XS5
33	P142529	38	A	X33	S22	XS31
34	P153105	38	A	X34	S23	XS32
35	P178869	38, 32	A	X11	S2	XS5
36	P236463	38	A	X1	S1	XS1
37	P267187	33	A	X1A	S1	XS1A
38	P271454	80, 38	A	X1C	S1	XS1C
39	E312893/0	38	A	X1	S1	XS1
40	P328130	33, 3.0	A	X35	S25	XS34
41	P366159	38	A	X14	S1	XS2

Ribotyping. Genomic DNA was extracted by the method of Wilson (29). Approximately 2 μg was digested with *Pvu*II (Boehringer Mannheim) and electrophoresed, together with digoxigenin-labelled *Hin*dIII digest of bacteriophage λ (Boehringer Mannheim), on a 0.8% agarose (Med EEO; Sigma, Poole, United Kingdom) gel. The DNA was transferred to Hybond N hybridization membranes as described above and hybridized to a 550-bp *rrnB* probe (3) prepared by PCR amplification and incorporating 11-dUTP–digoxigenin (Boehringer Mannheim) label. Immunological detection was done by standard protocols (Boehringer Mannheim), and restriction fragment patterns were compared visually. The level of discrimination provided was assessed by calculation of Simpsons' index as described by Hunter and Gaston (5).

PFGE. The method of strain preparation was essentially that described by Powell and colleagues (15). Chromosomal DNA contained in the agarose plugs was digested with 10 to 20 U of *Xba*I, *Not*I (Boehringer Mannheim), *Avr*II (*Bln*I),

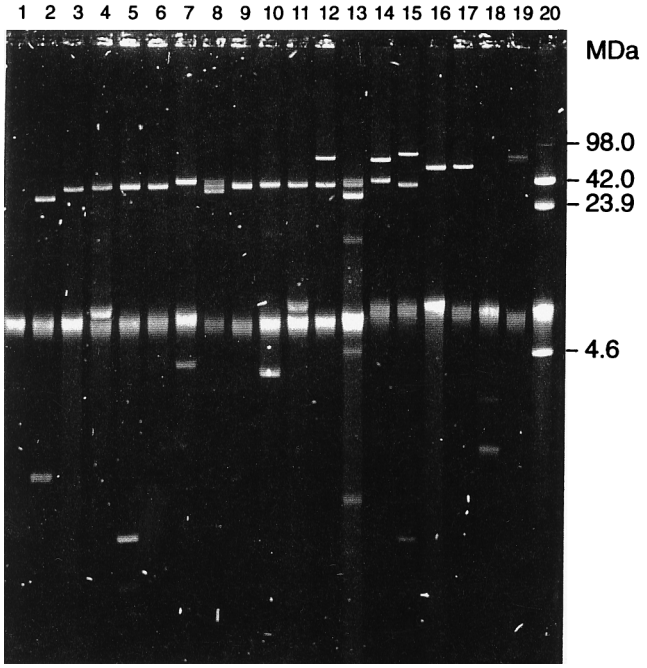

FIG. 1. Plasmid profiles of selected *S. enteritidis* PT reference strains showing the range of different types observed. Lanes: 1, P221267 (*S. enteritidis* PT 1a); 2, P328130 (PT 40); 3, E2187 (PT 4); 4, P367587 (PT 1c); 5, P316257 (PT 5b); 6, E2468 (PT 8); 7, E1949 (PT 19); 8, P138532 (PT 20a); 9, P72580 (PT 21); 10, P100613 (PT 25); 11, P125363 (PT 28); 12, P332932 (PT 6b); 13, P310001 (PT 24a); 14, P104204 (PT 30); 15, P271454 (PT 38); 16, E2109 (PT 11); 17, P106583 (PT 9a); 18, E2408 (PT 6a); 19, E3945 (PT 10); 20, *E. coli* 39R861.

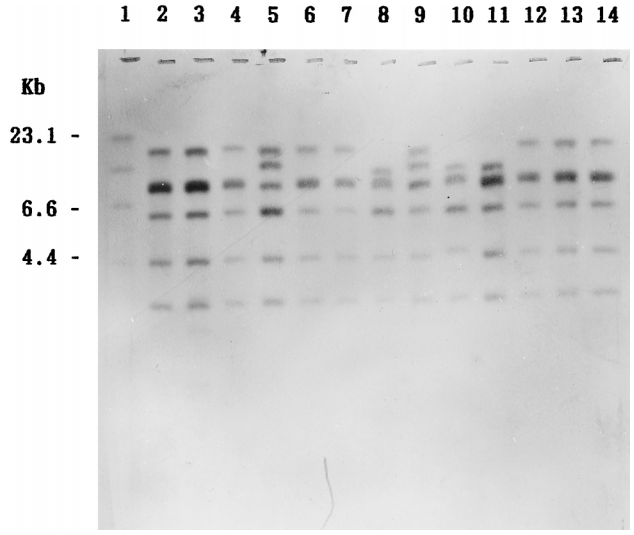

FIG. 2. *Pvu*II-derived 16S rRNA gene profiles of *S. enteritidis* PT reference strains selected on the basis of their different PFGE profiles showing the limited variation between the three ribotypes. Lane 1 shows *Hin*dIII-digested, Digoxigenin-labelled λ marker; lanes 2 to 14 show *Pvu*II digests. Lanes: 2, P367587 (*S. enteritidis* PT 1c); 3, E2187 (PT 4); 4, P316257 (PT 5b); 5, E2408 (PT 6a); 6, P278053 (PT 8a); 7, P312425 (PT 9c); 8, E2387 (PT 14); 9, P118526 (PT14b); 10, P106583 (PT 9a); 11, E2109 (PT 11); 12, P310001 (PT 24a); 13, P328130 (PT 40); 14, P366159 (PT 41).

*Spe*I, or *Nhe*I (New England Biolabs, Hitchin, United Kingdom). PFGE was performed with CHEF DRII systems (Bio-Rad, Hemel Hempstead, United Kingdom) in 0.5× Tris-borate-EDTA. DNA macrorestriction fragments were resolved on 1.0% agarose gels (PFGE certified; Bio-Rad). Lambda ladders, comprising 48.5-kb concatemers (Sigma), were used as size standards. Electrophoresis conditions used as standard in this study were 6 V/cm for 40 h. Pulse times were ramped from 15 to 40 s during the run for *Xba*I-generated macrorestriction fragments. Runs comprising 4.8 V/cm for 66 h ramped at 10 to 100 s were also employed in some cases to ensure that alterations to very high molecular weight fragments were not overlooked. The preferred pulse time employed for a 40-h PFGE analysis of *Spe*I macrorestriction fragments was 5 to 20 s.

The preparation of DNA from selected strains was repeated, and preparations of all strains were digested and electrophoresed under the same conditions on at least two occasions to assess the reproducibility of the method and the stabilities of strains. Macrorestriction fragment patterns were compared visually in order to obtain schematic representations of all profiles observed. PFPs were assigned to types in accordance with those obtained in a previous study (15). Simpson's index of discrimination was calculated as described above (5). Selected gels were blotted onto nylon membranes and hybridized to Digoxigenin-labelled *spvC*, as described above, in order to determine the macrorestriction fragments encoding the *S. enteritidis* SSP.

RESULTS

Plasmid profile and *spv* analysis. Plasmid masses, in megadaltons, obtained for each *S. enteritidis* PT reference strain are shown in Table 1. Of the 60 PT reference strains, 42 carried the 38-MDa *S. enteritidis* SSP, and of these 42 strains, 13 carried additional plasmids. Five PT reference strains were plasmid free, and the remainder carried at least one plasmid of 33 MDa or greater (Table 1 and Fig. 1). The reference strain for PT 6 (P99327) carried a 50-MDa plasmid, which hybridized to *spvC*, plus additional plasmids of 4.6 and 2.6 MDa, a profile which differed from that previously reported (24). Plasmids of approximately 59, 4.0, and 3.0 MDa were observed for the PT 6a reference strain, E2408 (Table 1). Both the 59-MDa plasmids of PTs 6a, 9a, 9b, 11, and 20 and the 33-MDa plasmids of PTs 9c, 37, and 40 hybridized to *spvC*. In contrast, the single 65-MDa plasmid carried by the reference strain of PT 10 (E3945) failed to hybridize to this probe, as did 65-MDa plasmids carried in addition to the 38-MDa SSP in the PT 5a and PT 22 reference strains.

Ribotyping. Three distinct *Pvu*II profiles were observed among the 60 *S. enteritidis* PT reference strains examined after hybridization with the 16S *rrnB* riboprobe (Fig. 2). Ribotyping with *Pvu*II as the sole restriction enzyme was not particularly helpful in discriminating within *S. enteritidis*, as the majority (50 of 60) of the reference strains belonged to *Pvu*II type A (Table 1 and Fig. 2, lanes 2 to 4, 6, 7, and 12 to 14); five strains generated each of the other ribotypes, B (Fig. 2, lanes 5 and 9) and C (Fig. 2, lanes 8, 10, and 11). The numerical index of discrimination (DI) for *Pvu*II ribotyping of the 60 PT reference strains was low (DI = 0.259).

PFGE analysis and identification of profile types. (i) *Xba*I. PFGE permitted the resolution of *Xba*I macrorestriction fragments of the 60 PT reference strains into 28 distinct types (Fig. 3). *Xba*I PFPs typically comprised 13 to 16 resolvable bands (usually 14) between 40 and 600 kb under the conditions used in the study (Fig. 4A), correlating well with the *Xba*I genomic cleavage map of *S. enteritidis* SSU7998, which revealed 16 fragments ranging from 19 to 900 kb in size (9). The predominant *Xba*I profile was identical to PFP 1 described by Powell et al. (15) and was accordingly designated PFP X1 (Fig. 4A, lane 2). A variation of PFP X1, designated PFP X1A, from which a single 57-kb fragment representing the 38-MDa plasmid was absent (Fig. 4A, lane 5), was observed for three strains, of which two (P221267 and P124191) were plasmid free. The 57-kb *Xba*I fragment corresponded to the 38-MDa plasmid encoding the common virulence region, which contains a single *Xba*I site, and appeared as a linear fragment of approximately 57 kb after resolution by PFGE. The PT 37 reference strain (P267187) carried a single 33-MDa plasmid, which was not visible by PFGE analysis under the conditions employed. In contrast, for the reference strains of *S. enteritidis* PTs 6 and 30,

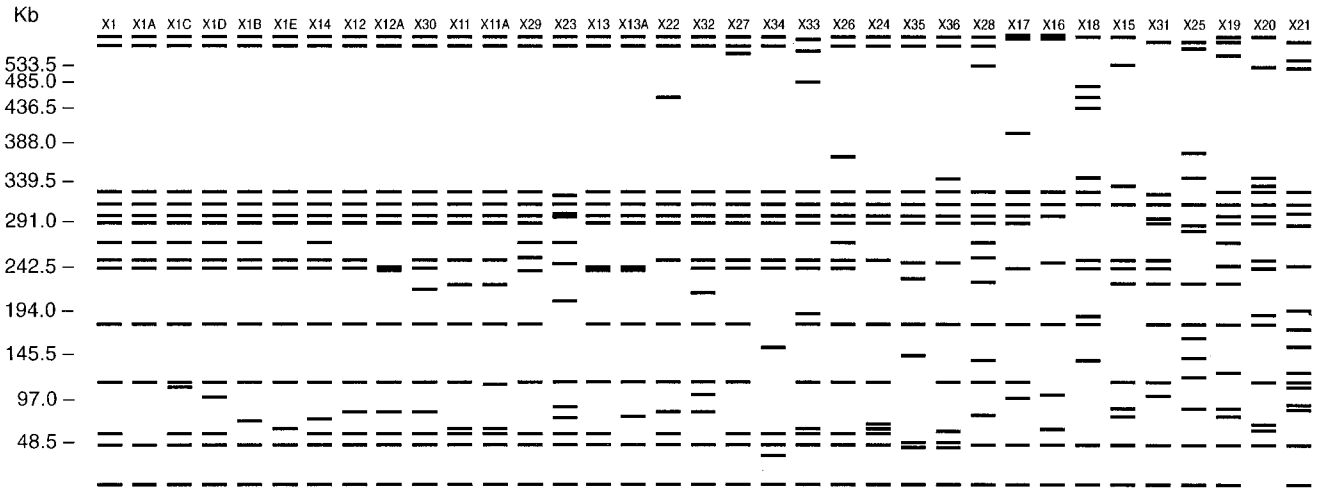

FIG. 3. Schematic representation of all 35 *Xba*I-generated macrorestriction profiles, including subtypes, resolved by PFGE for the 60 PT reference strains of *S. enteritidis*. The PFPs above the columns are correlated with strains in Table 1.

the 57-kb *Xba*I fragment was absent but plasmid-associated fragments of approximately 70 (PFP X1B) and 63 kb (PFP X1E) were generated, corresponding to the observed carriage of plasmids of 50 and 45 MDa, respectively (Table 1 and Fig. 4A, lane 4). The 70-, 63-, and 57-kb *Xba*I fragments, corresponding to the 50-, 45-, and 38-MDa plasmids, contained *spv*, as all hybridized to the *spvC* probe (not shown). The *Xba*I-generated PFPs X1, X12, X11, and X1A were the most commonly observed profiles among the *S. enteritidis* PT reference strains; PFP X1 was generated by the type strains of *S. enteritidis* PTs 1c, 4, 4a, 5a, 5b, 7a, 14b, 21, 24, 25, 27, 31, 36, and 39; PFP X12 was generated by PTs 2, 8, 8a, 13a, 23, and 28 (X12A by PT 7); PFP X11 was generated by PTs 1, 1b, 32, and 35 (X11A by PT 5); and PFP X1A was generated by reference strains of PTs 1a, 29, and 37 (Table 1). There were two instances of reference strains generating PFPs which were almost identical to those of an already-defined type but which differed by the appearance of a single *Xba*I-generated band in each case, which was reproducible for different preparations of the same strains and did not appear to be plasmid associated (not shown). The affected PTs were designated subtypes of the PFP from which they are likely to have been derived (PFP X11A from X11 and PFP X12A from X12 [Table 1]). PFPs X13 and X13A were differentiated by a positional change of a single plasmid-associated band (57 to 63 kb). The discrimination index calculated for *Xba*I macrorestriction profiles of *S. enteritidis* PTs analyzed by PFGE was 0.874.

(ii) *Spe*I. Macrorestriction fragments of the 60 *S. enteritidis* PT reference strains generated by *Spe*I were resolved into 25 individual profiles by PFGE by using pulse times of 5 to 20 s

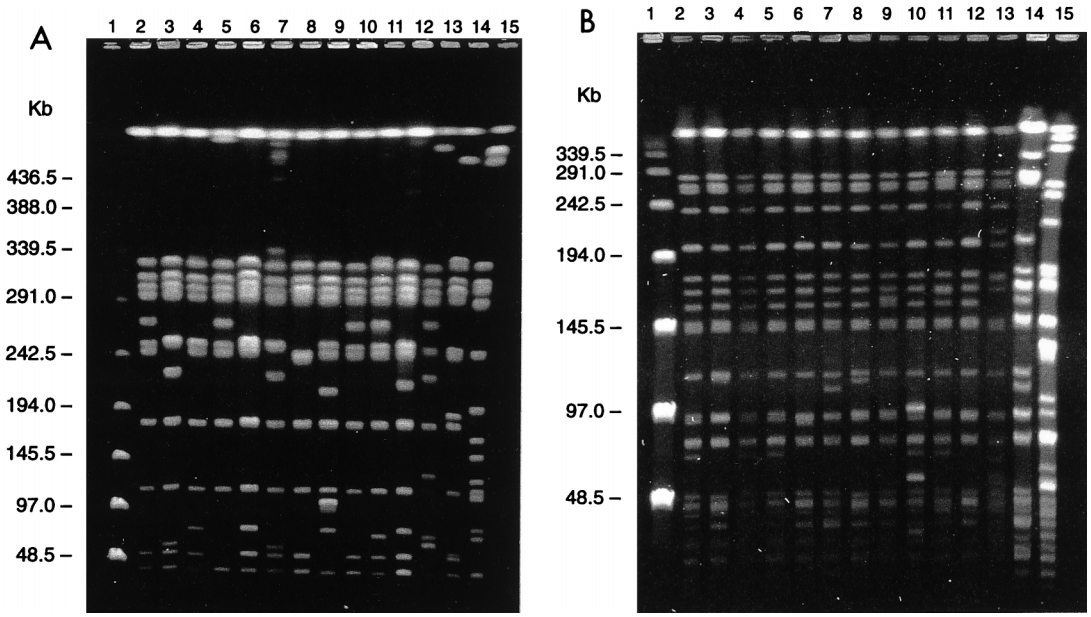

FIG. 4. PFGE analysis of *Xba*I (A)- and *Spe*I (B)-digested genomic DNA from *S. enteritidis* PT reference strains showing both similar and unique PFGE types. Lanes: 1, λ 48.5-kb ladder; 2, E2187 (*S. enteritidis* PT 4); 3, E2331 (PT 1); 4, P104204 (PT 30); 5, P221267 (PT 1a); 6, E2468 (PT 8); 7, P66040 (PT 3); 8, P135293 (PT 32); 9, E3945 (PT 10); 10, P122530 (PT 27); 11, P89448 (PT 18); 12, P84357 (PT 22); 13, E2109 (PT 11); 14, P138678 (PT 11a); 15, P187803 (PT 11b).

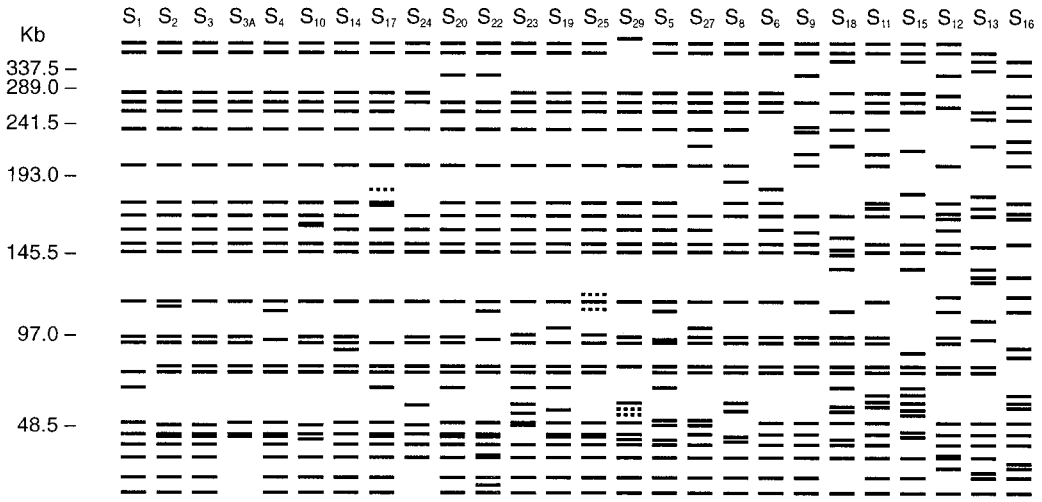

FIG. 5. Schematic representation of the 26 *Spe*I macrorestriction profiles, including one subtype, resolved by PFGE for the 60 PT reference strains of *S. enteritidis*. The PFPs above the columns are correlated with strains in Table 1. Broken lines correspond to faint but reproducible *Spe*I-generated bands.

(Fig. 4B and 5). Differences in profiles ranged from the loss of a single fragment of 70 to 75 kb to profiles which comprised five common resolvable bands in a macrorestriction profile which typically comprised 21 clearly resolvable bands of between 30 and 450 kb, some of which were observed as doublets (Fig. 4B).

Two *Spe*I PFPs predominated among the 60 *S. enteritidis* PT reference strains (Table 1 and Fig. 4B). The most common, PFP S1, was generated by *S. enteritidis* PTs 1a, 1c, 4, 4a, 5a, 5b, 6b, 7a, 12, 14b, 17, 18, 21, 24, 25, 29, 30, 36, 37, 38, 39, and 41 (Fig. 4B, lanes 2 and 5), while PFP S3 was produced by PTs 2, 8, 13a, 15, 22, 23, and 28 (Fig. 4B, lanes 6 and 12). The reference strains of *S. enteritidis* PTs 1, 1b, and 5 generated PFP S2 (Fig. 4B, lane 3). These three profiles were very similar, differing by the presence or absence of one or two bands. A single band of approximately 90 kb in PFP S1 appeared as a doublet in PFP S2; however, PFP S2 lacked a fragment of approximately 70 kb which was present in PFP S1 (Fig. 4B, lanes 2 and 3). Additional differences were observed in the migration of one fragment of the doublet of approximately 98 kb and one of 55 kb; these differences were found to be reproducible. PFP S3 differed from PFP S2 by the apparent loss of a 70-kb fragment, and PFP S3A was similar to PFP S3 but did not generate *Spe*I fragments below 40 kb. There did not appear to be a single *Spe*I-generated fragment correlating with the 57-kb fragment visible on *Xba*I-generated PFPs, corresponding to the presence of the 38-MDa *S. enteritidis* SSP. This was confirmed by hybridization of *Spe*I-digested DNA from strains of *S. enteritidis* with Digoxigenin-labelled *spvC* probe. The calculated discrimination index for *Spe*I-generated PFGE profiles of the 60 PT reference strains was 0.870, making this enzyme marginally less discriminatory than *Xba*I.

(iii) Combined *Xba*I and *Spe*I profiles. When the results of macrorestriction with *Spe*I and *Xba*I were combined there were six groups comprising two or more type strains of *S. enteritidis* PTs generating identical combined profiles. The remaining PT type strains generated unique combined PFPs. The largest group comprised 12 *S. enteritidis* PTs (1c, 4, 4a, 5a, 5b, 7a, 14b, 21, 24, 25, 36, and 39) and displayed a PFP XS1 macrorestriction profile, resulting from the combination of the predominant *Xba*I- and *Spe*I-generated profiles (Table 1). The second most prevalent combined type (PFP XS7) was generated by five PT type strains (PTs 2, 8, 13a, 23, and 28) and resulted from a combination of PFP X12 and PFP S3 (Table 1). There was a significant amount of overlap between PFPs generated by *Xba*I and *Spe*I, resulting in only a small increase in the number of types (to 32) and a corresponding improvement in discrimination (DI = 0.902).

(iv) Other restriction endonucleases. Macrorestriction analysis of *S. enteritidis* with *Not*I and *Nhe*I generated too many fragments below 100 kb for practical analysis, particularly as fragments below 70 kb were poorly resolved and could comprise digested extrachromosomal DNA. In contrast, *Avr*II, an isoschizomer of *Bln*I, generated a maximum of 10 resolvable fragments, compared with the 12 *Bln*I fragments reported for the genomic cleavage map of *S. enteritidis* SSU7998 (9), but did not offer improved discrimination over that of *Xba*I.

Reproducibility. Minor variations between different preparations of the same strains were not observed within the confines of the study, with the exception of a weak fragment of >700 kb appearing in occasional preparations (not shown). This was considered to be due to the presence of incompletely digested DNA and was usually removed by further treatment with proteinase K and subsequent washing steps.

Reduction of the electrophoresis time, from 64 to 40 h, with a corresponding increase in voltage, was applied in order to reduce the overall time required to generate a PFP. This was achieved, but with loss of resolution of very large fragments (>600 kb). However, profiles containing differences in bands of this size also had variations in smaller fragments.

Comparison of PFGE, ribotyping, and plasmid profile types. *S. enteritidis* PT reference strains generating the predominant PFGE combined type, PFP XS1, carried the 38-MDa SSP. Some strains were observed to carry additional plasmids (Table 1), none of which showed homology to *spvC*. With a single exception, this group of strains generated the predominant *Pvu*II ribotype (A). Only P118526 (PT 14b) generated ribotype B, which differed by the increase in size (from approximately 8.8 to 10.5 kb) of the second largest *Pvu*II-generated band (Fig. 2, lane 9). Strains belonging to the second most common combined PFP (XS7) generally carried a single plasmid of 38 MDa. Only P125363 (PT 28) carried an additional plasmid, of 8 MDa, which did not appear to affect either the ribotype or PFP. Of the *S. enteritidis* PT strains found to be plasmid-free

(PTs 1a, 11b, 16, 26, and 29), only those of PTs 1a and 29 generated PFP XS1A. The remaining strains belonging to this group generated unique *Xba*I and *Spe*I PFPs.

S. enteritidis PT reference strains generating ribotypes B and C did not carry the 38-MDa SSP and, with a single exception (PT 14b), generated combined PFPs unique to each strain (Table 1). Reference strains generating *Pvu*II ribotype B varied in plasmid profiles (Table 1). In contrast, four of the five strains generating *Pvu*II ribotype C possessed a single 59-MDa plasmid which showed homology to *spvC*, while P187803 (PT 11b) was plasmid free (Table 1).

DISCUSSION

Genotypic analysis of *Salmonella* by molecular typing methods has proved to be helpful in the characterization of strains from a range of *Salmonella* serovars (4, 11–16, 19–21, 26, 27). In this study, the 38-MDa SSP was not common to all *S. enteritidis* PTs but was present in the reference strains of most of the PTs commonly causing human infection. Notable exceptions were the reference strains of PT 6 (P99327) and PT 6a (E2408), each of which carried a plasmid of >38 MDa but also carried the common virulence region. However, examination of recent human strains belonging to PTs 6 and 6a indicates the presence of the 38-MDa SSP and the absence of the 59-MDa plasmid in the majority of strains (16, 16a). Although plasmid analyses have not been included in combination with other genotyping methods in previous studies involving typing of *S. enteritidis*, it was useful to correlate the presence of particular plasmids with fragments obtained by PFGE analyses. Typically, plasmids were observed as intensely stained fragments on PFGE gels, although this was less apparent for the 57-kb *Xba*I fragment corresponding to the *S. enteritidis* SSP.

This study has extended the molecular typing of *S. enteritidis* to 60 PTs. Plasmid-free strains did not hybridize to *spvC*, and the *Xba*I profiles were distinguished by the absence of a 57-kb fragment, which when present hybridizes to *spvC*. A corresponding fragment was not detected in *Spe*I macrorestriction profiles analyzed by PFGE, and it is presumed that the lack of a *Spe*I site in the SSP allows it to remain supercoiled and hence to migrate off the end of the gel. The results have provided a useful indicator as to how plasmids may affect PFGE profiles.

Although plasmid analysis appeared to exhibit a variety of profiles, the majority contained the 38-MDa *S. enteritidis* SSP, and many of the differences observed were due to carriage of additional small plasmids. In this study plasmids in addition to the 38-MDa SSP failed to hybridize to the *spvC* gene probe, and it was concluded that they were unrelated. Carriage of 4.0-MDa plasmids, as found in the type strains of *S. enteritidis* PTs 6a, 19, and 25, has been associated with resistance to ampicillin (3). The plasmids of 33, 50, 59, and 65 MDa carried by PT type strains not possessing the 38-MDa SSP hybridized to the *spvC* probe, generating *Xba*I fragments of 50, 75, 85, and 100 kb, respectively. This suggests that they are related to the SSP and contrasts with the findings of Liebisch and Schwartz (8), which suggested that large plasmid bands do not account for the RFLP detected by PFGE. The reference strain of PT 6 originally contained plasmids of 65, 38, 2.6, and 1.0 MDa (24), compared with the 50-, 4.0-, and 2.6-MDa plasmids observed for the same strain in this study. It may be that a segment of DNA has been lost from the 65-MDa plasmid and taken up by the 38-MDa SSP, thereby increasing its size to 50 MDa (Table 1). The 50-MDa plasmid was observed to hybridize to *spvC*, confirming the presence of the common virulence region in this plasmid. This study also reports the presence of a single 38-MDa SSP, corresponding to a 57-kb *Xba*I fragment, in the reference strain of PT 3 (P66040), a finding in contrast with those of Threlfall et al. (24), who observed a 59-MDa plasmid in the same strain. Although useful for strain discrimination in some outbreaks involving *S. enteritidis*, plasmid profiling does not generally convey strain-specific information.

Genotypic diversity between phenotypically related PTs. PFGE analysis demonstrates that some *S. enteritidis* PT reference strains generating similar phage typing reactions are genotypically distinct. This observation was most pronounced for reference strains of *S. enteritidis* PTs 11, 11a, and 11b for both *Xba*I and *Spe*I macrorestriction fragments (Fig. 4, lanes 13 to 15). The reference strains of PT 11 and 11a *Xba*I PFPs (X19 and X20) contained 14 band differences, for a total of 13 to 14 resolvable bands of ≥40 kb (Fig. 4A, lanes 13 to 15). The *Spe*I PFPs (S11 and S12) for the same strains resulted in 12 differences, for a total of 16 to 17 resolvable bands of ≥48.5 kb, where 8 bands were common to both PFPs (Fig. 4B, lanes 13 to 15). The *Spe*I PFP generated by the PT 11b reference strain (S13) shared six common fragments with each of the S11 and S12 PFPs, with between 9 and 17 band differences observed. Five *Xba*I fragments of ≥40 kb were observed to be shared between the PFPs of PTs 11a and 11b, and four were shared between PFPs of PTs 11 and 11b.

Fifteen band differences were identified between the *Xba*I macrorestriction profiles of PTs 14 and 14b and between 8 and 10 differences were identified between the *Xba*I PFPs of PTs 9, 9a, 9b, and 9c. Similarly, between 6 and 10 band differences were observed in the PFPs of the PT 6, 6a, and 6b reference strains. The corresponding *Spe*I profiles of these nine PT reference strains revealed between 4 and 10 band differences from a maximum of 18 resolvable bands of ≥48.5 kb. Such observations indicate that strains grouped according to phenotypic reactions may be genotypically diverse.

Less extensive variation in *Xba*I and *Spe*I macrorestriction profiles was observed between the reference strains of PTs 13 and 13a and for PTs 7 and 7a (up to three band differences), indicating a closer genotypic relationship. The type strains of PTs 1 and 1b shared both *Xba*I and *Spe*I PFPs. The *Spe*I PFPs generated by PTs 1a and 1c were indistinguishable from each other but differed in the positions of two bands from those of PTs 1 and 1b, while the *Xba*I PFPs differed by the absence of the 57-kb SSP. The type strains of *S. enteritidis* PTs 24 and 24a and those of PTs 20 and 20a revealed three and four band differences, respectively. In contrast, the type strains of PTs 4 and 4a shared both *Xba*I and *Spe*I PFPs (Table 1) and hence also shared a combined macrorestriction profile (PFP XS1). Minor differences in a single fragment size, leading to the designation of PFPs X11A, X12A, and S3A, were reproducible for different preparations of the same strains. Such variation may have resulted from a slight shift in migration of the fragment, the significance of which is unclear.

Several strains generating different phage reactions shared a common genotype. This was especially noticeable for the nine PT reference strains generating identical combined *Xba*I and *Spe*I macrorestriction profiles (PFP XS1). Although the phage reactions of the reference strains of *S. enteritidis* PTs 4 and 4a appeared to be similar, those of the reference strains of PTs 12, 21, and 36 and PTs 4, 5a, and 7a were markedly different. Only the PT 5a reference strain, which carried a 65-MDa plasmid in addition to the 38-MDa SSP, generated a distinct plasmid profile. Additional plasmids of this size have not been associated with changes to phage lysis patterns (22). The predominance of PFPs X1 and S1, generated by 14 and 22 PT reference strains, respectively, even among those type strains not commonly implicated in human infection suggests that this genotype may confer an as yet unknown selective advantage.

Small differences in a single fragment size, leading to PFPs X11A (PT 5) and X12A and S3A (PT 7), were reproducible for different preparations of the same strains. The altered fragments may have resulted from a shift in migration, the significance of which has not yet been evaluated.

The results of this study correlate well with those of previous studies by Olsen and colleagues (14), who reported that the convergence of PTs occurred between those which generated the same *Sma*I ribotype and the same *Not*I PFGE and *Pst*I RFLP profiles, although some generated different IS*200* profiles. Type strains from several PTs not previously typed by molecular methods were included in the present investigation. Of these, PTs 1c, 5a, 5b, 7a, 36, and 39 were indistinguishable from the predominant combined PFP XS1, ribotype A. Only PTs 1c, 5a, and 5b carried plasmids in addition to the 38-MDa SSP. *Xba*I and *Spe*I were chosen, as their discrimination between fragments of >48 kb was greater than that of other enzymes (*Not*I, *Nhe*I, and *Avr*II) evaluated. Olsen and colleagues (14) employed *Not*I and reported 10 different macrorestriction profiles for 33 *S. enteritidis* PT reference strains. However, the PT 16 reference strain (E866) was refractory to digestion by this enzyme. In addition, the numerous small *Not*I fragments of <100 kb were excluded for discriminatory purposes, as they were not clearly resolved. This study employed *Xba*I, generating 28 PFPs, with a further four subtypes of the predominant PFP X1, and *Spe*I, generating 25 PFPs for the 60 *S. enteritidis* PT type strains examined. This does not include additional PFPs identified for clinical isolates of *S. enteritidis* (15, 15a, 16). Furthermore, no strains were found to be refractory to digestion by the enzymes chosen for this study. A recent study demonstrated that ribotyping and plasmid profiling provided insufficient discriminatory power for epidemiological subdivision of a set of 31 unrelated strains of *S. enteritidis* of several different PTs (8). PFGE analysis provided improved typing of these strains; however, more than one enzyme was required to separate strains belonging to PTs 1, 4, 6, 7, and 8 (8). PTs of *S. enteritidis* predominantly causing infection in the United Kingdom include PTs 1, 4, 6, 6a, 8, and 13. By using PFGE analysis of macrorestriction fragments, the PT reference strains can be successfully differentiated. However, studies in this (16) and in other (14, 27) laboratories have demonstrated that some strains causing infection generate PFPs which may be markedly different from those observed for that particular PT reference strain, suggesting that strains causing infections in humans have a higher degree of genotypic homogeneity than is indicated by phage typing.

The level of discrimination permitted by combined *Xba*I and *Spe*I macrorestriction fragment analysis by PFGE (DI = 0.902) is superior to that obtained by ribotyping (DI = 0.259) and correlates well with a recent study of 31 isolates of *S. enteritidis*, for which an overall discrimination index for PFGE, calculated for a combination of three different enzymes, was 0.815 (8).

In this study we have addressed the framework of *Xba*I and *Spe*I macrorestriction fragments, analyzed by using PFGE for *S. enteritidis* by characterization of the 60 defined PT reference strains. The results suggest that PFGE analysis is potentially a valuable tool for the characterization of *S. enteritidis*. Furthermore, by combining results obtained for two or more enzymes, the discriminatory power of the method may be enhanced for several of the most common PTs causing infections in humans.

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